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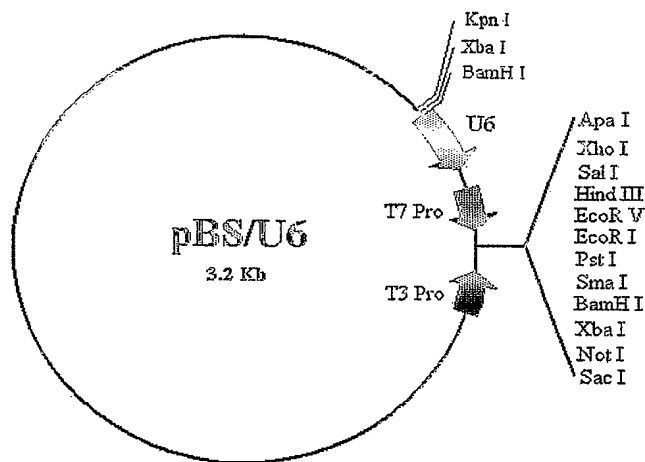
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(54) Title: RNA INTERFERENCE BASED METHODS AND COMPOSITIONS FOR INHIBITING HBV GENE EXPRESSION



(57) Abstract: The present invention provides methods  
for attenuating a target gene of hepatitis B virus (HBV)  
in a host cell using a double stranded RNA (dsRNA),  
which comprises a nucleotide sequence that hybridizes  
under stringent conditions to a nucleotide sequence of the  
HBV target gene.

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## RNA INTERFERENCE BASED METHODS AND COMPOSITIONS FOR INHIBITING HBV GENE EXPRESSION

### FIELD OF THE INVENTION

5            This invention relates to methods and compositions for attenuating expression of target genes of hepatitis B virus in host cells or animals using RNA interference (RNAi) mediated process.

### BACKGROUND OF THE INVENTION

10            Hepatitis B virus (HBV) infection is one of the most common viral diseases to human, and is a major cause of chronic viral hepatitis and liver cirrhosis distributed world widely. There are estimated 200-300 million people infected with HBV worldwide. In Southeast Asia and parts of Africa, the carriers are up to 20% of the population.

15            HBV is a partially double-stranded circular DNA virus of 42 nm diameter and belongs to a group of hepatotropic DNA virus (hepadnaviruses). The virus contains an outer coat and an inner core. The viral coat is composed mainly of hepatitis B surface antigen (HBsAg), which plays a central role in the diagnosis of HBV infection. The inner core contains hepatitis B core antigen (HBcAg), DNA polymerase/reverse  
20 transcriptase and the genome (Table 1). The genome is 3,200 bp (Blum, H.E., von Weizsacker, F., Wieland, S., Offensperger, S., and Offensperger, W.B. (1995) Adv. Drug Delivery Rev. 17, 321-331).

Table 1. Function of genes in HBV genome.

Gene*	Function	
pre-Surface 1	Encodes HBsAg, major diagnosis marker.	
pre — Surface 2		
Surface		
pre — Core		Encodes e antigen (HBeAg), a

Core	Encodes core antigen (HBcAg), a major structure protein of inner core.	major seromarker for high viral replication.
P-gene	Encodes viral DNA polymerase/reverse transcriptase, important for viral replication.	
X—gene	Encodes for x antigen (HBxAg), unknown function.	

\*P: polymerase 1-1626 (P-1) and 2310-3215 (P-2); HBsAg: 1-838 and 2851-3215; HBeAgCore: 1817-2455; X-protein: 1377-1841. The number represents the position of the gene in the genome.

5 HBV infection is a major cause of chronic viral hepatitis and liver cirrhosis worldwide, and is a major cause for converting to liver cancer. This virus has been characterized in great detail and can be identified by serological and molecular techniques. However, numerous mutations in HBV have been detected in patients with both acute and chronic HBV infection, and the mutations have been accumulated over  
10 time. This mutation has made the treatment of HBV infection more difficult.

RNA interference is the process of sequence-specific, posttranscriptional gene silencing that has become an active field in the antiviral research of mammals, *e.g.*, HIV (Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M. J., Ehsani, A., Dalvaterra, P. and Rossi, J. (2002) *Nature Biotechnology*, 19, 500-505) and HCV (Kapadia, S. B.,  
15 Brideau-Andersen, A. and Chisari, F. V. (2003) *Proc. Natl. Acad. Sci. USA*, 100, 2014-2018).

RNAi is an antiviral mechanism initially found in plants, *Caenorhabditis elegans* and *Drosophila*. It is known as post-transcriptional gene silencing (PTGS). RNAi has a highly gene-specific effect that functions through double strand RNA. It specifically  
20 reduces the expression of the gene with sequence homology to dsRNA. In this process, dsRNA is initially recognized and cleaved into 21-23-bp fragments called siRNA by an enzyme called Dicer with RNase III activity (Cullen, B. R. (2002) *Nat. Immunol.* 3, 597-599; Hannon, G. J. (2002) *Nature* 418, 244-251; Tuschl, T. (2001) *ChemBiochem* 2, 239-245; Sharp, P. A. (2001) *Genes Dev.* 15, 485-490; Moss, E. G. (2001) *Curr. Biol.* 11, 25 R772-R775); then the siRNA fragments specifically bind with a multicomponent

nuclease and form a complex known as the RNA-induced silencing complex (RISC); finally the RISC hybridizes to the target mRNA with a complementary region, and the target mRNA is cleaved in the middle of the 21- to 23-bp region (Cullen, B. R. (2002) Nat. Immunol. 3, 597-599; Hannon, G. J. (2002) Nature 418, 244-251; Tuschl, T. (2001) 5 Chembiochem 2, 239-245; Sharp, P. A. (2001) Genes Dev. 15, 485-490; Moss, E. G. (2001) Curr. Biol. 11, R772-R775). Thus the mRNA will not be functional.

RNAi also exists in mammalian cells (Cullen, B. R. (2002) Nat. Immunol. 3, 597-599; Yu, J. Y., DeRuiter, S. L. and Turner, D. L. (2002) Proc. Natl. Acad. Sci. USA 99, 6047-6052; Brummelkamp, T. R., Bernards, R. and Agami, R. (2002) Science 10 296,550-553; Sui, G., Soohoo, C., Affarell, B., Gay, F., Y. and Forrester, W. C. (2002) Proc. Natl. Acad. Sci. USA 99, 5515-5520). Recently, RNAi technology has been widely used in the study of many human diseases especially those caused by viruses. Present studies of RNAi in mammalian cells demonstrated that the expression of both exogenous genes delivered by DNA transfection and endogenous genes can be 15 suppressed by the delivery of siRNA (Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl. T. (2001) Nature 411(6836), 494—498; Garrus, J.E., von Schwedler, K., Pornillos, O.W., Morham, S.G., Zavitz, K.H., Wang, H.E., Wettstein, S.A., Stray, K.M., Cote, M., Rich, R.L., Myszka, D.G., Sundquist, W.I. (2001) Cell 107, 55-65). For example, it can suppress the expressions of HCV (hepatitis C virus), HIV-1 20 (human immunodeficiency virus-1), FHV (flock house virus), Rous sarcoma virus, dengue virus and poliovirus et al. (Kapadia, S.B., Brideau-Andersen, A. and Chisari, F.V. (2003) Proc. Natl. Acad. Sci. (USA) 100, 2014-2018). Most of these viruses are RNA-virus while HBV is a partially double strand DNA virus.

25

## SUMMARY OF THE INVENTION

The present invention provides a method for attenuating expression of target genes of hepatitis B virus (HBV) in a host cell, which method comprises providing a double stranded RNA (dsRNA) sequence in a host cell in an amount sufficient to attenuate expression of HBV target genes, said dsRNA sequence comprising a nucleotide

sequence that hybridizes under stringent conditions to a nucleotide sequence of a HBV target gene.

In another aspect, the present invention provides a pharmaceutical composition for preventing or treating HBV infection, which pharmaceutical composition comprises:

- 5 a) a double stranded RNA (dsRNA) sequence in an amount sufficient to attenuate expression of HBV target genes in a host cell, said dsRNA sequence comprising a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of a HBV target gene; or b) a double stranded DNA (dsDNA) sequence in an amount sufficient to attenuate expression of HBV target genes, said dsDNA sequence comprising  
10 a nucleotide sequence that is transcribed in a host cell to become a dsRNA sequence that hybridizes under stringent conditions to a nucleotide sequence of a HBV target gene.

In another aspect, the present inventions provides a kit for preventing or treating HBV infection, which kit comprises, an above-described pharmaceutical composition and an instruction for using said pharmaceutical composition for preventing or treating  
15 HBV infection in a mammal.

In another aspect, the present invention provides an isolated oligonucleotide sequence, which: a) hybridizes, under high stringency, with an oligonucleotide sequence, or a complementary strand thereof, that is set forth in Table 3; or b) has at least 90% identity to an oligonucleotide sequence, or a complementary strand thereof, that is set  
20 forth in Table 3.

The present invention also provides a vector comprising an isolated oligonucleotide sequence described above.

The present invention also provides a cell which comprises a vector of the invention.

25 The present invention also provides a pharmaceutical composition for preventing or treating HBV infection, which pharmaceutical composition comprises an isolated oligonucleotide sequence described herein .

The present invention also provides a pharmaceutical composition for preventing or treating HBV infection, which pharmaceutical composition comprises a vector of the  
30 invention described herein.

In some embodiments of the invention, the nucleotide sequence of the HBV target gene used for designing a dsRNA is in the coding region of the target gene.

### BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 illustrates a plasmid map of pBS/U6 for the cloning of siDNA. siDNA was cloned into *Apal* and *EcoRI* sites, and GFP cassette was inserted into *SmaI* site of pBS/U6.

Figure 2 illustrates a scheme of construction of siDNA-pBS/U6-GFP showing the location of siDNA and GFP, and the design of siDNA oligos forming hairpin structure.

10 Figure 3 illustrates visible (left panel) and fluorescence (right panel) images of HepG2.2.15 cells transfected with siDNA-pBS/U6-GFP.

Figure 4 illustrates the production of HBsAg from HepG2.2.15, and HepG2.2.15 transfected with pBS/U6-GFP and siDNA-1—7, respectively. HBsAg levels produced by cells transfected with siDNA-1 and -7 were significantly reduced comparing to those  
15 by HepG2.2.15 or HepG2.2.15 transfected with pBS/U6-GFP. After each transfection, cells expressing GFP were sorted by FACS and cultivated in two plates from which samples were taken for HBsAg-ELISA measurement. ELISAs were performed in triplicates. Data on siDNA-2 were from an independent experiment.

Figure 5 illustrates the mRNA quantification by real-time PCR using cDNA from  
20 cells as the PCR template. The siRNA measured were siRNA-1, -2 and -7; and HepG2.2.15 transfected with pBS/U6-GFP was used as the control. cDNAs were prepared by reverse transcription using the total RNA isolated from the cells, which had been treated with RNase-free DNase. To eliminate the contamination of chromosome DNA brought from the RNA which was used to amplify the cDNA, the same amount of  
25 RNA were used as the template in real-time PCR. Serial dilutions of templates of both HepG2.2.15 and GAPDH (Gyceraldehyde-3-Phosphate Dehydrogenase), the housekeeping gene, was used as the standard. The primers used in the real-time PCR were 5'- ATG GAG AAC ATC ACA TCA GGA TTC C -3' (upstream) (SEQ ID NO:1) and 5'- AAA GAA AAC AGA AAC CC ATA TGT AAA TT -3' (downstream) (SEQ ID  
30 NO:2 for HBV HBsAg; and 5'-CGG ATT TGG TCG TAT TGG G-3' (upstream) (SEQ

ID NO:3) and 5'-TCT CGC TCC TGG AAG ATG G-3' (downstream) (SEQ ID NO:4) for GAPDH. Experiments were performed in duplicates and the inhibition rates were calculated with the data obtained from two independent experiments.

Figure 6 illustrates the melting temperature of the real-time PCR product analyzed by LightCycler (Roche Applied Sciences, Mannheim, Germany). Single peak demonstrated the specificity of the PCR and reliability of the data to be used for the calculation of inhibition rate.

### DETAILED DESCRIPTION OF THE INVENTION

In many eukaryotes, expression of nuclear-encoded mRNA can be strongly inhibited by the presence of a double-stranded RNA (dsRNA, also termed siRNA) that is homologous to a certain part of the exon sequence in the mRNA. This mechanism is termed RNA interference, or RNAi. This invention is based on the discovery that a double stranded RNA (dsRNA) sequence that is homologous to a HBV target gene, when expressed in a host cell, attenuates expression of HBV genes expressed in the host cell. The methods and compositions provided by the invention can be used to prevent or treat HBV infections.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

#### A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications referred to herein are incorporated by reference in their entirety. If a definition set forth in this section is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein incorporated by reference, the definition set forth in this section prevails over the definition that is incorporated herein by reference.

As used herein, "a" or "an" means "at least one" or "one or more."

As used herein, a "double stranded RNA (dsRNA) sequence" or "siRNA" refers a double stranded RNA (dsRNA) molecule that is capable of attenuating the expression of a gene with which it shares homology. In some embodiments, the dsRNA molecule is a hairpin comprising a sense region, a loop region and an antisense region complementary to the sense region. The stem segment of the hairpin contains sequences that share homology with the target gene. In other embodiments, the dsRNA comprises two distinct RNA molecules that are non-covalently associated to form a duplex.

As used herein, "hairpin structure" refers to a polynucleotide or nucleic acid that contains a double-stranded stem segment and a single-stranded loop segment wherein the two polynucleotide or nucleic acid strands that form the double-stranded stem segment is linked and separated by the single polynucleotide or nucleic acid strand that forms the loop segment. The "hairpin structure" can further comprise 3' and/or 5' single-stranded region(s) extending from the double-stranded stem segment.

As used herein, "attenuating expression" refers to reduction or decrease in the level of mRNA and/or protein product from a target gene by at least about 10%, *e.g.*, about 20%, about 40%, about 60%, about 70%, about 80%, about 90%, or more, as compared to a control.

As used herein, a "target gene" refers a targeted nucleic acid sequence, the expression of which is attenuated in the present invention by dsRNA.

As used herein: "stringency of hybridization" in determining percentage mismatch is as follows:

- 1) high stringency: 0.1 x SSPE (or 0.1 x SSC), 0.1% SDS, 65°C;
- 2) medium stringency: 0.2 x SSPE (or 1.0 x SSC), 0.1% SDS, 50°C (also referred to as moderate stringency); and
- 3) low stringency: 1.0 x SSPE (or 5.0 x SSC), 0.1% SDS, 50°C.

It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

## **B. Attenuating hepatitis B virus genes in a host cell**



In one aspect, the present invention is directed to a method for attenuating expression of a target gene of hepatitis B virus (HBV) in a host cell, which method comprises providing a double stranded RNA (dsRNA) sequence in a host cell in an amount sufficient to attenuate expression of HBV target genes, said dsRNA sequence  
5 comprising a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of said HBV a target gene.

The double-stranded structure may be formed by a single self-complementary RNA strand (a hairpin structure) or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced  
10 in an amount which allows delivery of at least one copy per cell. Higher doses (*e.g.*, at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications.

The dsRNA can be synthesized *in vitro* or *in vivo* and delivered to the host cell using any known techniques capable of resulting in the desired attenuation of the target  
15 gene expression. The dsRNA used to attenuate expression of a target gene of HBV may be directly introduced into the host cell, for example, injected into the cell.

Alternatively, the dsRNA sequence is provided in the host cell by introducing a double stranded DNA (dsDNA) sequence into the host cell and the dsDNA sequence is transcribed into a dsRNA sequence in the host cell. The dsDNA sequence can be  
20 constructed in an expression vector which is delivered into the host cell. For example, expression vector can be a plasmid or a viral based vector. Any known viral or non-viral based delivery of the expression vector can be used.

Viral-based vectors, such as retrovirus vectors and adeno-associated virus vectors can be used as a delivery system for the transfer of exogenous genes into a host cell.  
25 These vectors provide efficient delivery of genes into the host cell, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host cell. Viral-based vectors for delivery of a desired polynucleotide and expression in a desired cell are well known in the art. Exemplary viral-based vehicles include recombinant retroviruses (*see, e.g.*, PCT Publication Nos. WO 90/07936; WO 94/03622; WO  
30 93/25698; WO 93/25234; WO 93/11230; WO 93/10218; WO 91/02805; U.S. Patent Nos.

5, 219,740 and 4,777,127; GB Patent No. 2,200,651; and EP 0 345 242), alphavirus-based vectors (*e.g.*, Sindbis virus vectors, Semliki forest virus (ATCC VR-67; ATCC VR-1247), Ross River virus (ATCC VR-373; ATCC VR-1246) and Venezuelan equine encephalitis virus (ATCC VR-923; ATCC VR-1250; ATCC VR 1249; ATCC VR-532)), adenovirus-derived vectors (*see, e.g.*, Berkner et al. (1998) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155), and adeno-associated virus (AAV) vectors (*see, e.g.*, PCT Publication Nos. WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655). Administration of DNA linked to killed adenovirus as described in Curiel, Hum. Gene Ther. (1992) 3:147 can also be employed.

Non-viral delivery vehicles and methods can also be employed, including, but not limited to, polycationic condensed DNA linked or unlinked to killed adenovirus alone (*see, e.g.*, Curiel, Hum. Gene Ther. (1992) 3:147); ligand-linked DNA (*see, e.g.*, Wu, J. Biol. Chem. (1989) 264:16985); eukaryotic cell delivery vehicles cells (*see, e.g.*, U.S. Patent No. 5,814,482; PCT Publication Nos. WO 95/07994; WO 96/17072; WO 95/30763; and WO 97/42338) and nucleic charge neutralization or fusion with cell membranes. Naked DNA can also be employed. Exemplary naked DNA introduction methods are described in PCT Publication No. WO 90/11092 and U.S. Patent No. 5,580,859. Liposomes that can act as gene delivery vehicles are described in U.S. Patent No. 5,422,120; PCT Publication Nos. WO 95/13796; WO 94/23697; WO 91/14445; and EP 0524968. Additional approaches are described in Philip, Mol. Cell Biol. (1994) 14:2411, and in Woffendin, Proc. Natl. Acad. Sci. (1994) 91:1581.

Successful introduction of the vectors into the host cell can be monitored using various known methods. For example, a marker gene contained in the vector, such as a green fluorescent protein (GFP) or any antibiotics or drug resistant genes can be used. The double stranded RNA and the marker gene may be expressed in the host cell transiently or stably after the vectors are introduced into the host cell.

The level of expression dsRNA can be controlled by regulatory sequences, and the vectors of the invention can include any regulatory sequences known in the art to act in a host cell, such as a mammalian cell. Exemplary regulator sequences include

promoters, enhancers, and other expression control elements. The regulatory sequences may be inducible promoters that depend on the presence of certain factors or tissue specific promoters (*e.g.*, liver specific).

The host cell which expresses a target gene of hepatitis B virus may be derived from or contained in any organism (*e.g.*, a mammal). The host cell can be an isolated cell or a cultured cell. The host cell may be comprised in a whole mammal. Examples of mammals include non-human mammal (*e.g.*, dog, cat, horse, pig, sheep, cattle, goat, rodent, hamster, mouse, rat, and primate) and human. The host cell expressing the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The host cell may be a stem cell or a differentiated cell. The host cell may be a cell derived from the liver of a mammal. The method of the invention can be used to prevent or treat HBV infection in the mammal.

Exemplary HBV target genes include pre-Surface 1, per-Surface 2, surface genes (encoding HBsAg), pre-Core, Core genes (encoding HBeAg), Core gene (encoding HBcAg), P gene and X gene (Table 1). Exemplary nucleotide sequence of a HBV target gene that can be used for designing a dsRNA nucleotide sequence includes conserved HBV sequences set forth in Table 2 (SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, or SEQ ID NO:11).

20

Table 2. Seven conserved HBV sequences used for siDNA design

	Conserved HBV sequences for siDNA design	nt	In the region of coded protein*
1	(404) TTC CTC TTC ATC CTG CTG CTA TGC CTC ATC TTC TT* (SEQ ID NO:5)	35	P-1 and HBsAg
2	(640) CCT ATG GGA GTG GGC CTC AG (SEQ ID NO:6)	20	P-1 and HBsAg
3	(1866)TTC AAG CCT CCA AGC TGT GCC TTG G (SEQ ID NO:7)	25	HBeAgCore
4	(2373)GAA GAA GAA CTC CCT CGC CTC GCA GAC	28	HBeAgCore and P-2

	G (SEQ ID NO:8)		
5	(247) GAG TCT AGA CTC GTG GTG GAC TTC TCT CAA TTT TCT AGG GG (SEQ ID NO:9)	41	P-1 and HBsAg
6	(1177) TGC CAA GTG TTT GCT GAC GCA ACC CCC AC (SEQ ID NO:10)	29	P-1
7	(1254) GGC TCC TCT GCC GAT CCA TAC TGC (SEQ ID NO:11)	24	P-1

\* The locations of coded protein are based on HBV AY090458 (GenBank accession number). P: polymerase 1-1626 (P-1) and 2310-3215 (P-2); HBsAg: surface antigen (LHBS, MHBS, HBsAg), 1-838 and 2851-3215; HBeAgCore: core antigen 1817-2455; X-protein: 1377-1841. (The numbers are the locations of genes in HBV sequences)

In some embodiments, the dsRNA sequence comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence set forth in Table 2 (SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, or SEQ ID NO:11).

In some embodiments, the dsRNA sequence comprises a pair of nucleotide sequences selected from the group consisting of siHBV-1, siHBV-2, siHBV-3, siHBV-4, siHBV-5, siHBV-6, siHBV-7 set forth in Table 3 (SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, or SEQ ID NO:18), and their complementary sequences.

Table 3. siRNA sequences that target HBV genome

	Target siRNA sequences in HBV genome	Location of siRNA
siHBV-1	5'-GCT GCT ATG CCT CAT CTT CT-3' (SEQ ID NO:12)	418 — 437*, P+HBsAg**
siHBV-2	5'-CCT ATG GGA GTG GGC CTC A-3' (SEQ ID NO:13)	640—658, P+HBsAg

siHBV-3	5'-AAG CCT CCA AGC TGT GCC T-3' (SEQ ID NO:14)	1869-1887, HBeAg
siHBV-4	5'-GAA GAA GAA CTC CCT CGC CT-3' (SEQ ID NO:15)	2373-2392, P+HBeAg+ HBcAg
siHBV-5	5'-GAC TCG TGG TGG ACT TCT CT-3' (SEQ ID NO:16)	254-273, P+HBsAg
siHBV-6	5'-GCC AAG TGT TTG CTG ACG CA-3' (SEQ ID NO:17)	1178-1197, P
siHBV-7	5'-GGC TCC TCT GCC GAT CCA TAC-3' (SEQ ID NO:18)	1254-1274, P

\*Location of the oligo in HBV genome;

\*\* P: polymerase 2310-3215(1)-1626; **HBsAg**: surface antigen 1-838 and 2851-3215(1); **HBeAg**: e antigen, 1817-2455; **HBcAg**: core, 1907-2455; **X-protein**: 1377-1841.

5 In some embodiments, the dsRNA sequence of the invention does not comprise nucleotide sequences set forth in Table 4 (SEQ ID NOS:19-29).

Table 4. List of siRNAs targeting HBV genome that were published in related publications.

Origin	siRNA sequences that target HBV genome	Location of siRNA
Nature Biotechnology, (2003) 21, 639-44	HBVU6no.1 5'-TCG TGG TGG ACT TCT CTC AAT TTT C-3' (SEQ ID NO:19)	257-281, P+ HBsAg
	HBVU6no.2 5'-CTC AGT TTA CTA GTG CCA TTT GTT C-3' (SEQ ID NO:20)	674-698, P+ HBsAg
	HBVU6no.3 5'-ATG ATG TGG TAT TGG GGG CCA	745-770, P+ HBsAg

	AGT C-3' (SEQ ID NO:21)	
	HBVU6no.4 5'-TGG CCA AAA TTC GCA GTC CCC AAC C-3' (SEQ ID NO:22)	304-328, P+ HBsAg
	HBVU6no.5 5'-TCC CCG TCT GTG CCT TCT CAT CTG C-3' (SEQ ID NO:23)	1548-1573, X-protein
	HBVU6no.6 5'-CCT AGA AGA AGA ACT CCC TCG CCT C-3' (SEQ ID NO:24)	2368-2393, P+ HBcAg
	HBVU6no.7 5'-AGA AGA TCT CAA TCT CGG GAA TCT C-3 (SEQ ID NO:25)	2423-2447, P+ HBcAg
<i>Hepatology</i> , (2003) 37, 764-70	5'-GGT CTT ACA TAA GAG GAC T-3' (SEQ ID NO:26)	1649-1667, X-protein
	5'-GAT CAG GCA ACT ATT GTG G-3' (SEQ ID NO:27)	2191-2209, HBcAg
	5'-GCC ATT CTC TGC TGG GGG G-3' (SEQ ID NO:28)	2075-2093, HBcAg
<i>FEBS Letters</i> , (2003) 543, 51-54	5'-CAT TGT TCA CCT CAC CAT ATT-3' (SEQ ID NO:29)	2137-2157, HBcAg

\*Location of the oligo in HBV genome;

\*\* **P**: polymerase 2310-3215(1)-1626; **HBsAg**: surface antigen 1-838 and 2851-3215(1); **HBcAg**: e antigen, 1817-2455; **HBcAg**: core antigen; 1907-2455; **X-protein**: 1377-1841.

5 In some embodiments, the expression vector (for example, a plasmid) that can be transcribed into the dsRNA sequence may further comprise a detectable marker in the host cell. An example of such detectable markers is a green fluorescent protein (GFP).

In some embodiments, the dsRNA or the dsDNA encoding the dsRNA comprise a nucleotide sequence of at least about 19, 20, or 21 nucleotides in length of the target

sequence. In some embodiments, the dsRNA or the dsDNA encoding the dsRNA are at least about 25, 50, 100, 200, 300, or 400 nucleotides in length of the target sequence.

In some embodiments, the dsRNA encoded by a dsDNA forms a hairpin structure.

5 In some embodiments, the expression of HBV target genes is attenuated by at least about 20%, 40%, 60%, 80%, 90%, or more, relative to the untreated host cell or a host cell treated with a dsDNA which does not encode a dsRNA correspond to a target gene. The expression of the HBV target genes can be measured at RNA level and/or protein level by any biochemical techniques such as RNA hybridization, PCR, Western  
10 blotting, enzyme linked immunosorbent assay (ELISA), and fluorescence activated cell sorting analysis (FACS).

### C. Oligonucleotides and vectors for the expression of siRNA

The present invention also provides an isolated oligonucleotide sequence, which:  
15 a) hybridizes, under high stringency, with an oligonucleotide sequence, or a complementary strand thereof, that is set forth in Tables 3; or b) has at least about 90% identity to an oligonucleotide sequence, or a complementary strand thereof, that is set forth in Tables 3.

In some embodiments, the oligonucleotide sequence includes a nucleotide  
20 sequence at least about 70%, 75%, 80%, 85%, 90%, or 95% identical to the oligonucleotide sequence, or a complementary strand, that is set forth in Table 3. In some embodiments, the oligonucleotide sequence comprises a nucleotide sequence, that is set forth in Table 3.

In some embodiments, the oligonucleotide sequence of the invention comprises  
25 DNA, RNA, PNA, or a derivative thereof.

The invention also provides an isolated double stranded oligonucleotide sequence, which comprises a pair of complementary oligonucleotide sequences of the isolated oligonucleotide sequence described herein. For example, the isolated double stranded oligonucleotide sequence may comprise a pair of nucleotide sequences set forth in Table  
30 3 and its complementary sequences.

In some embodiments, the double stranded oligonucleotide sequence is a dsDNA or a dsRNA.

The present invention also provides a vector comprising an isolated oligonucleotide sequence describe herein. The vector can be a plasmid or a viral vector.  
5 The vector can be introduced into a host cell and integrated into the host genome. The vector can also be constructed to permit it to be an extrachromosomal plasmid after being introduced into a host cell.

In some embodiments, the vector further comprises regulatory sequences that control the expression of the isolated oligonucleotide sequence. In some embodiments,  
10 the vector further comprises a transcription terminator.

In some embodiments, the vector further comprises a nucleotide sequence encoding a detectable marker (*e.g.*, a green fluorescent protein (GFP)) in the host cell.

The present invention also provides a cell, which cell comprises a vector described herein. The cell can be an isolated cell or a cultured cell. In some  
15 embodiments, the cell is comprised in a non-human whole mammal. In some embodiments, the cell stably expresses siRNA and/or other sequence(s) encoded by the vector. In some embodiments, the cell is a HepG2.2.15 cell.

#### **D. Pharmaceutical compositions**

20 In another aspect, the present invention provides a pharmaceutical composition for preventing or treating HBV infection, which pharmaceutical composition comprises:  
a) a double stranded RNA (dsRNA) sequence in an amount sufficient to attenuate expression of HBV target genes in a host cell, said dsRNA sequence comprising a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence  
25 of a HBV target gene; or b) a double stranded DNA (dsDNA) sequence in an amount sufficient to attenuate expression of HBV target genes, said dsDNA sequence comprising a nucleotide sequence that is transcribed in a host cell to become a dsRNA sequence that hybridizes under stringent conditions to a nucleotide sequence of a HBV target gene.

In another aspect, the present invention provides a pharmaceutical composition  
30 for preventing or treating HBV infection, which pharmaceutical composition comprises



an isolated oligonucleotide sequence, which: a) hybridizes, under high stringency, with an oligonucleotide sequence, or a complementary strand thereof, that is set forth in Tables 3; or b) has at least 90% identity to an oligonucleotide sequence, or a complementary strand thereof, that is set forth in Tables 3.

5 In another aspect, the present invention provides a pharmaceutical composition for preventing or treating HBV infection, which pharmaceutical composition comprises a vector comprising an isolated oligonucleotide sequence described herein.

In some embodiments, the pharmaceutical composition of the invention further comprises a pharmaceutically acceptable carrier or excipient. Pharmaceutically  
10 acceptable excipients are known in the art, and are relatively inert substances that facilitate administration of a pharmacologically effective substance. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolality, encapsulating agents, buffers, and skin penetration enhancers. Excipients as  
15 well as formulations for parenteral and nonparenteral drug delivery are set forth in Remington, The Science and Practice of Pharmacy 20th Ed. Mack Publishing (2000).

### E. Kits

The present invention provides a kit for preventing or treating HBV infection,  
20 which kit comprises any pharmaceutical composition described herein and an instruction for using said pharmaceutical composition for preventing or treating HBV infection in a mammal.

### EXAMPLES

25 In these examples, a double strand DNA which is homologous to conserved part of HBV mRNA and can form hairpin structure after transcription, was used as siDNA and cloned into plasmid pBS/U6. A GFP cassette containing nucleic-location-site (NLS) was also cloned into this siRNA expressing vector to monitor plasmid transfection. After transfecting these vectors into a HBV-positive stable cell line HepG2.2.15, the cells  
30 were sorted by fluorescence activated cell sorting (FACS) technique and tested for the

expression of HBV surface antigen (HBsAg). The control cells used to calculate the RNAi inhibition rate on HBsAg expression were HepG2.2.15 cells transfected with pBSU6-GFP plasmid, which did not contain siRNA sequences. After culturing the cells for 120 h, the HBsAg production of HepG2.2.15 by siRNA-1, -2, -3, -4, -5, -6, and -7 (measured by ELISA) were suppressed by 71.8%, 26.3% (result from an independent experiment), 20.2%, 35.2%, 26.8%, 17.0%, and 88.2%, respectively when compared to control cells.

Real-time PCR was performed to quantitate the mRNA level of HBV gene transcribed in HepG2.2.15, and the inhibition rates of HBV mRNA by RNAi were 60.8%, 25.3% and 84.7% for siRNA-1, -2 and -7, respectively, using HepG2.2.15 transfected with pBSU6-GFP as the negative control. These inhibition results were consistent with those of HBsAg assay (by ELISA) for siRNA-1, -2 and -7, respectively.

Further analysis with Northern blotting and Western immunoblotting is being performed to confirm inhibition at mRNA level. The oligos of the invention can be formulated as a drug to be delivered to the target tissue (liver) in human body as a gene therapy treatment.

### **Experimental procedures**

#### *HBV sequences for siRNA designation*

HBV sequences from 19 individuals were retrieved from GenBank™. Their GenBank accession numbers are AB076679, AB076678, AY090461, AY090460, AY090459, AY090458, AY090457, AY090456, AY090455, AY090454, AY090453, AY090452, E10905, NC\_003977, AB074756, AB074755, AB064316, AB064315 and AB064314, respectively. Homologous analysis of the above HBV DNA sequences was performed using an on-line DNA analysis tool <http://www.ebi.ac.uk/clustalw/>.

#### *Plasmid constructions for the expression of siRNA*

Plasmids that contain siDNA were constructed based on the plasmid pBS/U6 and expression of siRNA was under the control of U6 promoter (Figure 1). siDNA was inserted between *ApaI* and *EcoRI* sites of this plasmid (Figure 1).

A green fluorescent protein (GFP) cassette was also inserted into pBS/U6 at *SmaI* site. It was PCR amplified from pCR3.1-Uni (Invitrogen Life Technologies, CA, USA)

and contains the functional part of GFP of 753 bp and the adjacent P<sub>CMV</sub> promoter and BGH polyA transcription terminator.

A nucleic-location-site (NLS) was located in front of GFP, and its sequence was 5'-ACC ATG CAC CGC AGG AAG AAG AAG AGG AGA ACC-3' (SEQ ID NO:30), which was synthesized from BioAsia Biotechnology Ltd. (Shanghai, China). The constructed plasmid was designated siDNA-pBS/U6-GFP.

#### Cloning host

*E. coli* DH5 $\alpha$  was used as the siDNA cloning host. Ampicillin at 0.1 g/l was added for selected screening of cells containing pBS/U6 or its derivatives.

#### 10 Cell culture

HepG2.2.15 was used as the HBV-positive cell line for the transcription of pBS/U6 and its derivatives. Cells were cultured in DMEM (GIBCO) supplemented with 10% heat inactivated FBS at 37°C in a 5% CO<sub>2</sub> incubator.

#### Transfection

15 Transfections of siDNA-pBS/U6-GFP or pBS/U6 plasmids into HepG2.2.15 were performed using Effectene Transfection Reagent Kit (Qiagen, MD, USA) by ELISA according to the manufacture's instruction. Briefly, HepG2.2.15 cells of  $0.5 \times 10^5$  were seeded in a 6-well dish in 600  $\mu$ l medium 24 h before transfection, and the cells should be between 60-80% confluency before transfection complex was added. Plasmid of 0.4  $\mu$ g with a concentration of  $\geq 0.1 \mu\text{g}/\mu\text{l}$  was used in each transfection reaction. Cells were washed once with PBS 16 h after transfection.

#### Fluorescence analysis and FACS (Fluorescence-Activated Cell Sorting)

Fluorescence images of cells transfected with siDNA-pBS/U6-GFP were collected under microscopy LEICA MPS60 (Solms, Germany).

25 Cells expressing GFP were sorted 48 h after transfection and collected with FACSDiva (Becton Dickinson Bioscience, IOWA, USA). Then the cells were cultured in fresh medium.

#### Determination of HBV surface antigen

Supernatant of cell culture medium was used for HBsAg quantitation with HBsAg Quantitation Kit S-01 (YingKe Corp., Xiamen, China). Assays were performed in duplicates.

Real-time PCR to evaluate the HBsAg expression at mRNA level

5 Total RNA were isolated using RNeasy Mini Kit (Qiagen, MD, USA), and then treated with RNase-free DNase I (Amplification grade, Invitrogen, CA, USA).

cDNA was prepared by reverse transcription using M-MLV Reverse Transcriptase kit (Invitrogen, CA, USA). The dNTPs and Oligo(dT)<sub>6</sub> used in the reverse transcription were both from Promega (WI, USA). cDNA was quantitated by the  
10 absorbance at 260 nm.

Real-time PCR was performed in 20 ul volume on LightCycler (Roche Applied Sciences, Mannheim, Germany). The data from real-time PCR were analyzed by LightCycler Software Version 3. The inhibition rate of HBsAg expression was calculated according to Pfaffl M.W. (Pfaffl M.W. (2001) Nucleic Acids Res. 29: e45).

## Results

### Plasmid constructs

The genome sequences of HBV from different individuals vary a lot. To choose siDNA sequences targeting HBV, homology analysis was performed on HBV genome  
20 sequences from 19 individuals published in GenBank and obtained 8 conserved sequences that were over 19 nucleotides in length. We used seven of them as the siDNA candidates (Table 5).

Table 5. Seven conserved HBV sequences used for siDNA design

	Conserved HBV sequences for siDNA design	nt	In the region of coded protein*
1	(404) TTC CTC TTC ATC CTG CTG CTA TGC CTC ATC TTC TT* (SEQ ID NO:5)	35	P-1 and HBsAg
2	(640) CCT ATG GGA GTG GGC CTC AG (SEQ ID NO:6)	20	P-1 and HBsAg

3	(1866)TTC AAG CCT CCA AGC TGT GCC TTG G (SEQ ID NO:7)	25	HBeAgCore
4	(2373)GAA GAA GAA CTC CCT CGC CTC GCA GAC G (SEQ ID NO:8)	28	HBeAgCore and P-2
5	(247) GAG TCT AGA <i>CTC GTG GTG GAC TTC TCT</i> CAA TTT TCT AGG GG (SEQ ID NO:9)	41	P-1 and HBsAg
6	(1177)TGC CAA GTG TTT GCT GAC GCA ACC CCC AC (SEQ ID NO:10)	29	P-1
7	(1254)GGC <i>TCC TCT GCC GAT CCA TAC</i> TGC (SEQ ID NO:11)	24	P-1

\* The locations of coded protein are based on HBV AY090458 (GenBank accession number). P: polymerase 1-1626 (P-1) and 2310-3215 (P-2); HBsAg: surface antigen (LHBS, MHBS, HBsAg), 1-838 and 2851-3215; HBeAgCore: core antigen 1817-2455; X-protein: 1377-1841. (The numbers are the locations of genes in HBV sequences)

5

To construct siDNA, oligos of 19-nt, which had G/C 45-65%, were selected from the sequences in Table 5, and used to design 4 oligos, 1a, 1b, 2a and 2b (Table 6) for each siDNA. After annealing, "Oligo 1a" and "Oligo 1b" formed "GG" at 5'-end as a part of U6 promoter and a *HindIII* site at its 3'-end, and "Oligo 2a" and "Oligo 2b" formed a *HindIII* site at 5'-end and a "TTTTT" followed by an *EcoRI* site at its 3'-end. Then the double strand oligo, Oligo 1a/1b, was inserted between *ApaI* and *HindIII* sites of pBS/U6, and Oligo 2a/2b inserted between *HindIII* and *EcoRI* sites (as shown in Figure 2). In the construct, these two pairs of oligos were joined by *HindIII* site, and contained a Pol III transcription terminator "TTTTT" at the 3' end of "Oligo 2a/2b". Thus the transcription product of siDNA will fold back to form a hairpin joined by a 6-bp *HandIII* site.

15

Table 6. Four sets of siDNA were designed for the construction of siRNA expression vectors. Letters in bold indicated the sequences that were not homologous to HBV's.

20

"Oligo 1a" was complementary to "Oligo 1b", and "Oligo 2a" to "Oligo 2b" to form

double strands small DNAs, respectively. Fragments 1a/1b and 2a/2b can form a hairpin structure after transcription.

---

siHBV-1:

- 5 1. siHBV-1-1a (sense) 5' GGCTGCTATGCCTCATCTTCTA 3' (SEQ ID NO:31)
2. siHBV-1-1b (antisense) 5' AGCTTAGAAGATGAGGCATAGCAGCC 3' (SEQ ID NO:32)
3. siHBV-1-2 a (sense) 5' AGCTTAGAAGATGAGGCATAGCAGCCCTTTTGTG 3' (SEQ ID NO:33)
- 10 4. siHBV-1-2 b (antisense) 5' AATTCAAAAAGGGCTGCTATGCCTCATCTTCTA 3' (SEQ ID NO:34)

siHBV-2:

5. siHBV-2-1a (sense) 5' GGCCTATGGGAGTGGGCCTCAA 3' (SEQ ID NO:35)
- 15 6. siHBV-2-1b (antisense) 5' AGCTTTGAGGCCCACTCCCATAGGCC 3' (SEQ ID NO:36)
7. siHBV-2-2a (sense) 5' AGCTTTGAGGCCCACTCCCATAGGCCCTTTTGTG 3' (SEQ ID NO:37)
8. siHBV-2-2b (antisense) 5' AATTCAAAAAGGGCCTATGGGAGTGGGCCTCAA
- 20 3' (SEQ ID NO:38)

siHBV-3:

9. siHBV3-1a (sense) 5' GGAAGCCTCCAAGCTGTGCCTA 3' (SEQ ID NO:39)
10. siHBV-3-1b (antisense) 5' AGCTTAGGCACAGCTTGGAGGCTTCC 3' (SEQ ID NO:40)
- 25 11. siHBV-3-2a (sense) 5' AGCTTAGGCACAGCTTGGAGGCTTCCCTTTTGTG 3' (SEQ ID NO:41)
12. siHBV-3-2b (antisense) 5' AATTCAAAAAGGGAAGCCTCCAAGCTGTGCCTA 3' (SEQ ID NO:42)
- 30

## siHBV-4:

13. siHBV-4-1a (sense) 5' **GGAAGAAGA**ACTCCCTCGCCTA 3' (SEQ ID NO:43)  
14. siHBV-4-1b (antisense) 5' **AGCTTAGGCGAGGGAGTTCTTCTTCC** 3' (SEQ ID NO:44)  
5 15. siHBV-4-2a (sense) 5' **AGCTTAGGCGAGGGAGTTCTTCTTCCC TTTT**TG 3' (SEQ ID NO:45)  
16. siHBV-4-2b (antisense) 5' **AATTCAAAAAGGGAAGAAGA**ACTCCCTCGCCTA 3' (SEQ ID NO:46)
- 10 siHBV-5  
17. siHBV-5-1a (sense) 5' **GGACTCGTGGTGGACTTCTCTA** (SEQ ID NO:47)  
18. siHBV-5-1b (antisense) 5' **AGCTTAGAGAAGTCCACCACGAGTCC** (SEQ ID NO:48)  
19. siHBV-5-2a (sense) 5' **AGCTTAGAGAAGTCCACCACGAGTCCCTTTT**TG  
15 (SEQ ID NO:49)  
20. siHBV-5-2b (antisense) 5' **AATTCAAAAAGG**ACTCGTGGTGGACTTCTCTA (SEQ ID NO:50)

## siHBV-6

- 20 21. siHBV-6-1a (sense) 5' **GGCCAAGTGTTTGCTGACGCAA** (SEQ ID NO:51)  
22. siHBV-6-1b (antisense) 5' **AGCTTTGCGTCAGCAAACACTTGGCC** (SEQ ID NO:52)  
23. siHBV-6-2a (sense) 5' **AGCTTTGCGTCAGCAAACACTTGGCCCTTTT**TG (SEQ ID NO:53)  
25 24. siHBV-6-2b (antisense) 5' **AATTCAAAAAGGGCCAAGTGTTTGCTGACGCAA** (SEQ ID NO:54)

## siHBV-7

25. siHBV-7-1a (sense) 5' **GGCTCCTCTGCCGATCCATACA** (SEQ ID NO:55)  
30 26. siHBV-7-1b (antisense) 5' **AGCTTGTATGGATCGGCAGAGGAGCC** (SEQ ID

NO:56)

27. siHBV-7-2a (sense) 5' AGCTTGTATGGATCGGCAGAGGAGCCCTTTTGG  
(SEQ ID NO:57)

28. siHBV-7-2b (antisense) 5' AATTCAAAAAGGGCTCCTCTGCCGATCCATACA  
5 (SEQ ID NO:58)

To direct the expression of GFP into nucleic of the cell, a 33-nt NLS was cloned in front of GFP. The resulted construct designated siDNA-pBS/U6-GFP would express siDNA as well as GFP in nuclei as an intracellular marker for selection.

10 The successful cloning of siDNA into pBS/U6 was verified by DNA sequencing, and the cloning of GFP was confirmed by the visualization of green fluorescence under fluorescence microscopy.

#### Selection of cells expressing GFP

Cells expressing GFP were sorted by FACS and considered to contain siDNA  
15 (Figure 3). Over 95% of cells collected from FACS contained GFP indicating the high sorting efficiency.

#### Detection of HBsAg by ELISA

To evaluate RNAi effect on HBV expression, cells collected from FACS were cultivated and the supernatant was used in ELISA for HBsAg quantitation. Both  
20 HepG2.2.15 and HepG2.2.15 transfected with pBS/U6-GFP (did not contain siDNA) were used as the negative control for RNAi. The production of HBsAg was monitored every 24 h from cells transfected with each plasmid (Figure 4), and ELISA for HBsAg assay was performed in duplicates. Data in Figure 4 were the average of two independent transfection experiments.

25 Figure 4 showed HBsAg production from cells transfected with different siDNAs including pBS/U6-GFP. Supernatant of cell culture were taken at time intervals after cells sorted by FACS for HBsAg measurement. The results in Figure 4 indicated that production of HBsAg of HepG2.2.15 transfected with siDNA-1, siDNA-2, siDNA-3, siDNA-4, siDNA-5, siDNA-6, siDNA-7 had lower rates of HBsAg production  
30 comparing to HepG2.2.15 transfected with pBS/U6-GFP. Production of HBsAg of



HepG2.2.15 transfected with siDNA-1 and siDNA-7 were inhibited more significantly (71.8% and 88.2% inhibition rates at 120 h after sorting, respectively) than HepG2.2.15 transfected with other siDNAs. The inhibition rates of HepG2.2.15 transfected siDNAs at 120 h after cells were sorted by FACS are shown in Table 7.

5        Quantification of HBV mRNA by real-time PCR

To further evaluate the effect of RNAi, real-time PCR was performed to quantitate HBV mRNA transcribed in the presence of siRNA, and compared with the control. Figure 5 showed the mRNA quantification by real-time PCR using the cDNA of HBsAg as the template to amplify HBsAg fragment. The samples assayed were total  
10 RNA isolated from HepG2.2.15 cells transfected with siRNA-1, 2 and -7; HepG2.2.15; HepG2.2.15 transfected with pBS/U6-GFP. The signals of siRNA-7 and -1 showed up later in cycles than those of the controls and the rest of siRNAs, indicating lower levels of mRNA (of HBsAg). Also, the signal of the negative control using only RNA (not cDNA) as the template did not show up until after 30 cycles, indicating no chromosome  
15 DNA contamination in the RNA used for preparing cDNA, the template of real-time PCR. This also demonstrated that the signals in real-time PCR for the siRNAs were all amplified from their corresponding cDNAs eliminating the possibility of amplification from the chromosome DNA.

Results in Figure 6 showed the analysis of melting temperature ( $T_m$ ) on the  
20 product of real-time PCR which indicates the specific amplification of the target nucleotide. This results indicated that the real-time PCR performed amplified only one product and the data were reliable for the calculations of the inhibition rates of HBsAg expressions by different RNAi, which were shown in Table 7.

Results in Table 7 indicated that the inhibition rates on HBsAg expression  
25 measured on siRNA-1, -2 and -7 by ELISA were consistent with those by real-time PCR. RNAi-7 had the highest the inhibition rate on the expression of HBsAg showing the effect of RNAi technology used in the inhibition of HBV expression in human liver cells.

30    Table 7 Inhibition rates of HBsAg production by siDNA-1—7 at 120 h after cell sorting.

The rates were calculated using pBS/U6 transfected HepG2.2.15 as the control.

Transfections of HepG2.2.15 with siDNA-1 and -7 significantly inhibited HBsAg production of HepG2.2.15. Inhibition rates measured by real-time PCR were comparable with those done by ELISA. The inhibition rate of HBsAg transcription in real-time PCR was calculated according to Pfaffl, 2001 (Michael W. Pfaffl, 2001, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (9):e45).

HBsAg							
Inhibition rates* (%)	siDNA-1	siDNA-2**	siDNA-3	siDNA-4	siDNA-5	siDNA-6	siDNA-7
By ELISA	71.8±8.2	26.3±4.8	20.2±6.6	35.2±9.6	26.8±6.7	17.0±6.3	88.2±1.2
By real-time PCR	60.8±4.2	25.3±0.0	NA***	NA	NA	NA	84.7±1.6

\*Averages of two cell cultures after each FACS. ELISAs were performed in triplicates.

\*\*From an independent experiment.

\*\*\*Not available.

#### Screening for stable cell line expressing siRNA

To obtain a stable cell line expressing siRNA, cells collected from FACS were plated in fresh medium, and sorted by FACS again after 2-3 generations. After several times sorting, all the cells contained GFP by fluorescence microscopy. This cell line can be considered to be a GFP stable cell line, as well as siDNA containing stable cell lines.

#### Discussion

In vitro the active synthesized siDNA was usually 21-nt-long with symmetrical 2- to 3-nt 3' overhangs (Elbashir, S.M., Lendeckel, W. and Tuschl, T. (2001) Genes Dev. 15, 188-200). But in *C. elegans* and *Drosophila*, the siRNA could be either long dsRNA or

a hairpin diRNA (Tavernarakis, N., Wang, S.L., Dorovkov, M., Ryazanov, A. and Driscoll, M. (2000) Nat. Genet. 24, 180-183; Kennerdell, J.R. and Carthew, R.W. (2000) Nat. Biotechnol. 18, 96-898). To use pBS/U6 as the siRNA expression vector, in this study "GG" was added in the front of each 19-nt to resume "GGG" as a part of the  
5 transcription initiation site of U6 promoter and the resulted mRNA would form a hairpin.

The transcription of different genes of HBV results in only one mRNA for different proteins. Since the delivery of siDNA into liver cell results in the activation of mRNA degradation and inhibition of protein synthesis, the location of the conserved sequences in HBV genome for siDNA design should have no effect on RNAi. However,  
10 in our results in Figures 4 and 5, we observed clear differences in levels of inhibition of HBsAg production in the seven siDNA transfected cells. This indicated that the location of siDNA in each coding region of HBV may play roles in effectiveness of RNAi activity.

Since HBV is an easily mutated virus, we designed the siRNAs against the  
15 conserved parts of HBV genome sequences. Therefore, the RNAi effective siRNA-1 and -7 can be generally applied to the knockdown of HBV from different individuals.

Since siDNA and GFP were constructed on the same plasmid, cells expressing GFP was considered also expressing siRNA. However, not all the cells collected from FACS retained GFP over cultivation time, which indicated the lost of plasmid or the  
20 decreasing of GFP expression over time. These cells did not contribute to RNAi, which means that the results we obtained in Table 7 were conserved. Stable cell lines were also under selection by FACS.

Real-time PCR is a newly developed method to examine the amount of mRNA transcribed from certain genes. It can also be used to evaluate the HBsAg mRNA left in  
25 the cell after being digested by RNAi effect. The inhibition rates calculated on siRNA-1, -2 and -7 in Table 7 indicated the consistency of the two methods used, ELISA and real-time PCR. RNAi-7 had the highest degree of inhibition on the expression of HBsAg showing the effect of RNAi technology in inhibiting of HBV expression in human liver cells.

The above examples are included for illustrative purposes only and are not intended to limit the scope of the invention. Many variations to those described above are possible. Since modifications and variations to the examples described above will be apparent to those of skill in this art, it is intended that this invention be limited only by  
5 the scope of the appended claims.

### CLAIMS

1. A method for attenuating expression of target genes of hepatitis B virus (HBV) in a host cell, which method comprises providing a double stranded RNA (dsRNA) sequence in a host cell in an amount sufficient to attenuate expression of HBV target genes, said dsRNA sequence comprising a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of a HBV target gene.
2. The method of claim 1, wherein the dsRNA sequence is provided in the host cell by introducing a dsRNA sequence into the host cell directly.
- 10 3. The method of claim 1, wherein the dsRNA sequence is provided in the host cell by introducing a double stranded DNA (dsDNA) sequence into the host cell and the dsDNA sequence is transcribed into a dsRNA sequence in the host cell.
4. The method of claim 3, wherein the dsRNA sequence is introduced into the host cell via a plasmid comprising the dsDNA sequence.
- 15 5. The method of claim 1, wherein the host cell is an isolated cell or a cultured cell.
6. The method of claim 1, wherein the host cell is comprised in a whole mammal.
7. The method of claim 6, which is used to prevent or treat HBV infection in  
20 the mammal.
8. The method of claim 7, wherein the mammal is a non-human mammal.
9. The method of claim 7, wherein the mammal is a human.
10. The method of claim 1, wherein the HBV target gene is selected from the group consisting of pre-Surface 1, per-Surface 2 and surface genes (encoding HBsAg);  
25 pre-Core and Core genes (encoding HBeAg); Core gene (encoding HBcAg); P gene (encoding polymerase); and X gene.
11. The method of claim 1, wherein the nucleotide sequence of the HBV target gene is selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11.

12. The method of claim 1, wherein the dsRNA sequence comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence set forth in SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11.

5 13. The method of claim 1, wherein the dsRNA sequence comprises nucleotide sequences selected from the group consisting of siHBV-1, siHBV-2, siHBV-3, siHBV-4, siHBV-5, siHBV-6, and siHBV-7 set forth in Table 3.

14. The method of claim 1, wherein the dsRNA sequence does not comprise nucleotide sequences set forth in Table 4.

10 15. The method of claim 4, wherein the plasmid comprising the dsDNA sequence further comprises a nucleotide sequence encoding a detectable marker in the host cell.

16. The method of claim 15, wherein the detectable marker is a green fluorescent protein (GFP).

15 17. The method of claim 1, wherein the dsRNA sequence comprises at least about 19 to about 21 nucleotides in length of the target gene sequence or a complementary to the target gene sequence.

18. The method of claim 17, wherein the dsRNA sequence forms a hairpin structure.

20 19. The method of claim 1, wherein expression of the HBV target gene is attenuated by at least about 80%.

20. A pharmaceutical composition for preventing or treating HBV infection, which pharmaceutical composition comprises:

25 a) a double stranded RNA (dsRNA) sequence in an amount sufficient to attenuate expression of HBV target genes in a host cell, said dsRNA sequence comprising a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of a HBV target gene; or

b) a double stranded DNA (dsDNA) sequence in an amount sufficient to attenuate expression of HBV target genes, said dsDNA sequence comprising a nucleotide

sequence that is transcribed in a host cell to become a dsRNA sequence that hybridizes under stringent conditions to a nucleotide sequence of a HBV target gene.

21. The pharmaceutical composition of claim 20, further comprising a pharmaceutically acceptable carrier or excipient.

5        22. A kit for preventing or treating HBV infection, which kit comprises, a pharmaceutical composition of claim 20 and an instruction for using said pharmaceutical composition for preventing or treating HBV infection in a mammal.

23. An isolated oligonucleotide sequence, which:

a) hybridizes, under high stringency, with an oligonucleotide sequence, or a  
10 complementary strand thereof, that is set forth in Tables 3; or

b) has at least 90% identity to an oligonucleotide sequence, or a complementary strand thereof, that is set forth in Tables 3.

24. The isolated oligonucleotide sequence of claim 23, which comprises DNA, RNA, PNA or a derivative thereof.

15        25. The isolated oligonucleotide sequence of claim 23, which comprises a nucleotide sequence, or a complementary strand thereof, that is set forth in Tables 3.

26. An isolated double stranded oligonucleotide sequence, which comprises a pair of complementary oligonucleotide sequences of claim 23.

27. The isolated double stranded oligonucleotide sequence of claim 26, which is  
20 dsDNA or dsRNA.

28. A vector, which vector comprises an isolated oligonucleotide sequence of claim 23.

29. The vector of claim 28, which further comprises a nucleotide sequence encoding a detectable marker in the host cell.

25        30. The vector of claim 29, wherein the detectable marker is a green fluorescent protein (GFP).

31. The vector of claim 28, which is a plasmid.

32. A cell, which cell comprises a vector of claim 28.

33. The cell of claim 32, which is an isolated cell or a cultured cell.

30        34. The cell of claim 32, which is comprised in a non-human whole mammal.

35. The cell of claim 32, which stably expresses encoded siRNA and/or other sequence(s).

36. The cell of claim 32, which is a HepG2.2.15 cell.

37. A pharmaceutical composition for preventing or treating HBV infection,  
5 which pharmaceutical composition comprises an isolated oligonucleotide sequence of claim 23.

38. A pharmaceutical composition for preventing or treating HBV infection, which pharmaceutical composition comprises a vector of claim 28.



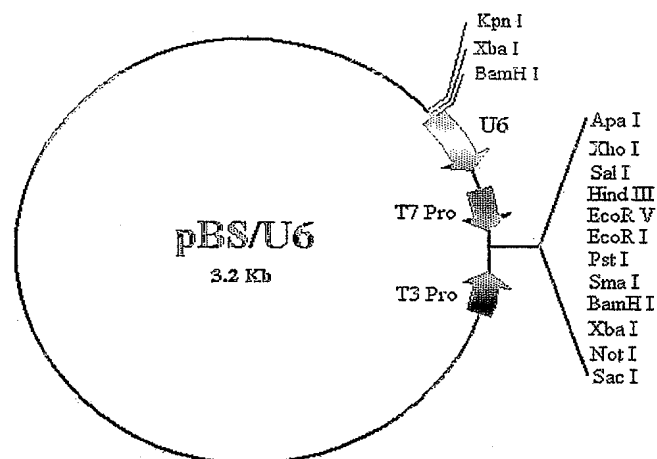


Figure 1

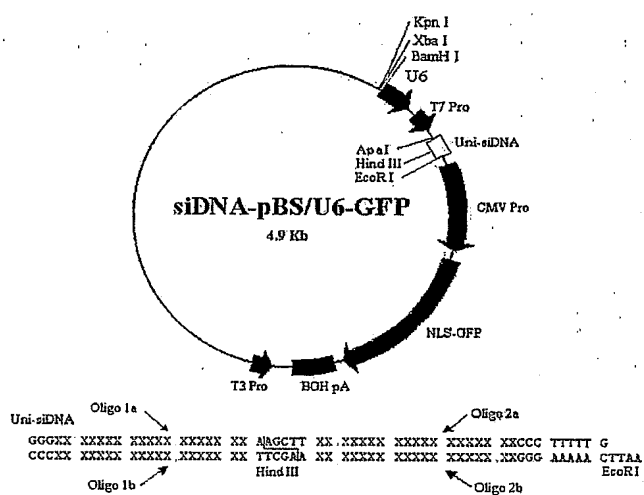


Figure 2

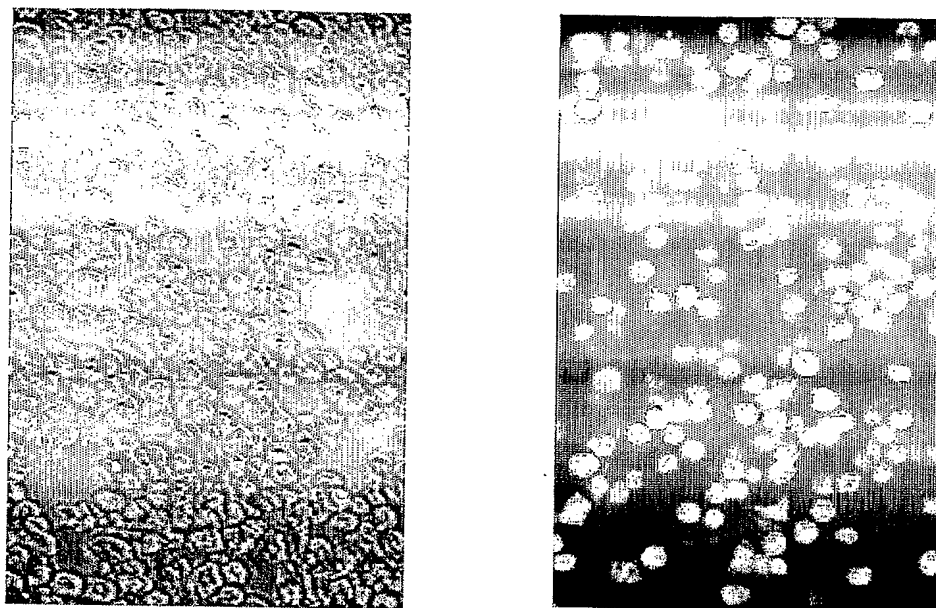


Figure 3

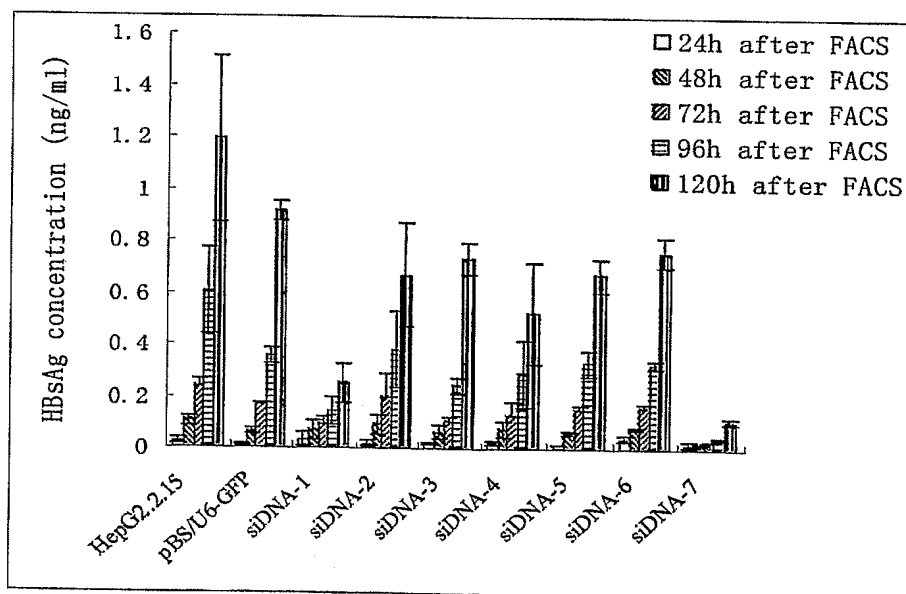


Figure 4

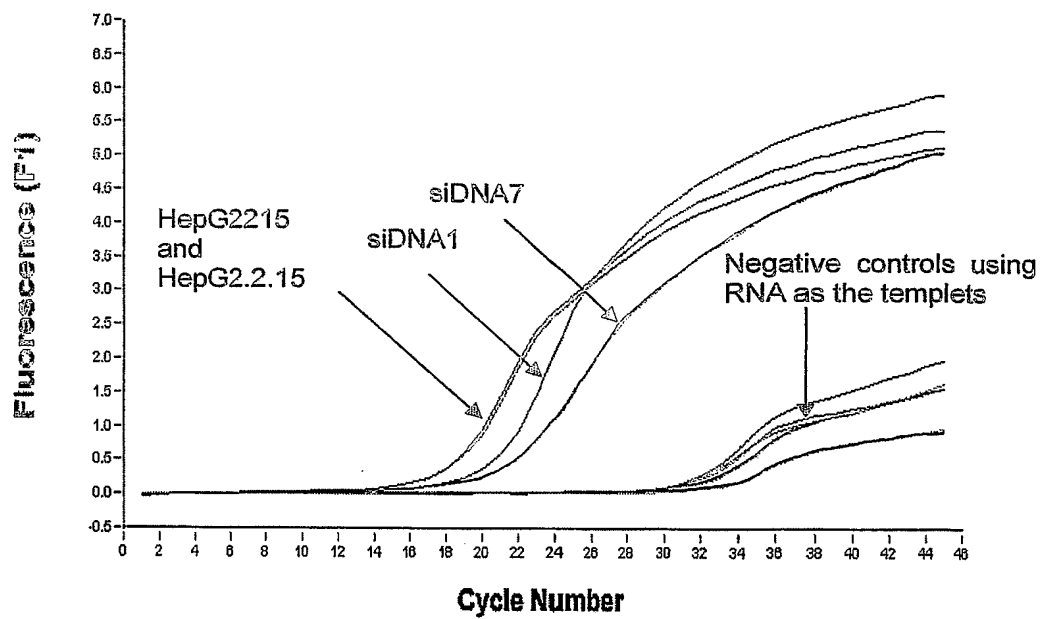


Figure 5

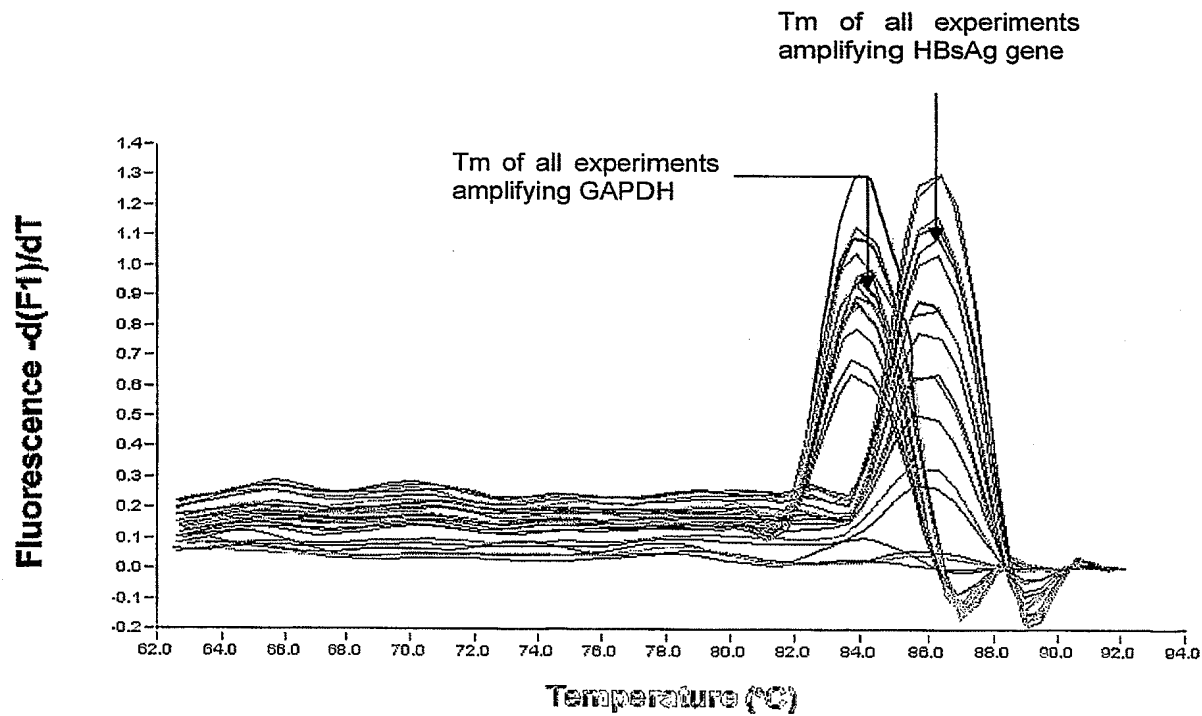


Figure 6

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CN03/00718

## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>7</sup> A61K31/70; A61K31/713; A61K48/00; A61P31/20; C07H21/00; C07H21/02; C07H21/04; C12N15/09  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>7</sup> A61K31/70; A61K31/713; A61K48/00; A61P31/20; C07H21/00; C07H21/02; C07H21/04; C12N15/09

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNPAT, EPOQUE(WPI), NCBI, CA, BA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9920641, ((SHAN-N) SHANGHAI INST BIOCHEMISTRY CHINESE ACAD, et al), 29, April, 1999, see the whole document	1-10, 20-22
A		11-19, 21-38
A	WO, A1, 02083908, ((CIEN-N) INST CIENTIFICO & TECNOLOGICO NAVARRA SA), 24, December, 2002, see the abstract	1-38
A	WO, A1, 9206693, ((FOXC-N) FOX CHASE CANCER CENT), 30, April, 1992, see the abstract	1-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L" document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 23 September 2003(23. 09.2003)	Date of mailing of the international search report 23 OCT 2003 (23. 10.03)
Name and mailing address of the ISA/CN 6 Xitucheng Rd., Jimen Bridge, Haidian District, 100088 Beijing, China Facsimile No. 86-10-62019451	Authorized officer Wei chunbao Telephone No. 86-10-62093435

Form PCT/ISA/210 (second sheet) (July 1998)

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
PCT/CN03/00718

Patent document cited in search report	Publication date	Patent family members	Publication date
WO, A1, 9920641	29-04-1999	JP-T- 2002511384T CN-A-1215058 AU-A-9528798 CN-A-1276794	16-04-2002 28-04-1999 10-05-1999 13-12-2000
WO, A1, 02083908	24-10-2002	NONE	
WO, A1, 9206693	30-04-1992	AU-A-8957591 EP-A1-0554376 JP-T-6502311T	20-05-1992 11-08-1993 17-03-1994